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(54) Title: THERAPEUTIC USE

(57) Abstract: An EGFR tyrosine kinase inhibitor is used in the manufacture of a medicament for use in (a) reducing the transformation of epithelial cells from a normal to a malignant state in an invasive breast cancer free human; and/or (b) reducing the transformation of epithelial cells from an intermediate state, between normal epithelium and malignant invasive epithelium, to a malignant state in an invasive breast cancer free human; and/or (c) causing substantial reversion of epithelial tissue back to a normal state from an intermediate state between normal epithelium and malignant invasive epithelium.

THERAPEUTIC USE

The present invention relates to the therapeutic use of
compounds which possess inhibitory activity against the
5 epidermal growth factor receptor (EGFR) tyrosine kinase enzyme.
In particular the invention relates to the use of such
compounds to inhibit the transformation of normal cells into
cancerous cells i.e. the compounds are cancer chemopreventative
agents.

10   In recent years it has been discovered that a cell may
become cancerous by virtue of the transformation of a portion
of its DNA into an oncogene i.e. a gene which, on activation,
leads to the formation of malignant tumour cells (Bradshaw,
Mutagenesis, 1986, 1, 91). Several such oncogenes give rise to
15 the production of proteins which are receptors for growth
factors. The growth factor receptor complex subsequently leads
to an increase in cell proliferation. It is known, for
example, that several oncogenes encode tyrosine kinase enzymes
and that certain growth factor receptors are also tyrosine
20 kinase enzymes (Yarden et al., Ann. Rev. Biochem., 1988, 57,
443; Larsen et al. Ann. Reports in Med. Chem. 1989, Chpt. 13).
      Receptor tyrosine kinases are important in the
transmission of biochemical signals which initiate cell
replication. They are large enzymes which span the cell
25 membrane and possess an extracellular binding domain for growth
factors such as epidermal growth factor (EGF) and an
intracellular portion which functions as a kinase to
phosphorylate tyrosine amino acids in proteins and hence to
influence cell proliferation. Various classes of receptor
30 tyrosine kinases are known (Wilks, Advances in Cancer Research,
1993, 60, 43-73) based on families of growth factors which bind
to different receptor tyrosine kinases. The classification
includes Class I receptor tyrosine kinases comprising the EGF
family of receptor tyrosine kinases such as the EGF, TGFα, NEU,
35 erbB, Xmrk, HER and let23 receptors, Class II receptor tyrosine
kinases comprising the insulin family of receptor tyrosine

kinases such as the insulin, IGFI and insulin-related receptor (IRR) receptors and Class III receptor tyrosine kinases comprising the platelet-derived growth factor (PDGF) family of receptor tyrosine kinases such as the PDGFαa, PDGFβb and
5 colony-stimulating factor 1 (CSF1) receptors.

It is known that Class I kinases such as the EGF family of receptor tyrosine kinases are frequently present in common human epithelial cancers such as breast cancer (Sainsbury et al., Brit. J. Cancer, 1988, 58, 458; Guerin et al., Oncogene
10 Res., 1988, 3, 21 and Klijn et al., Breast Cancer Res. Treat., 1994, 29, 73), non-small cell lung cancers (NSCLCs) including adenocarcinomas (Cerny et al., Brit. J. Cancer, 1986, 54, 265; Reubi et al., Int. J. Cancer, 1990, 45, 269; and Rusch et al., Cancer Research, 1993, 53, 2379) and squamous cell cancer of
15 the lung (Hendler et al., Cancer Cells, 1989, 7, 347), bladder cancer (Neal et al., Lancet, 1985, 366), oesophageal cancer (Mukaida et al., Cancer, 1991, 68, 142), gastrointestinal cancer such as colon, rectal or stomach cancer (Bolen et al., Oncogene Res., 1987, 1, 149), cancer of the prostate (Visakorpi
20 et al., Histochem. J., 1992, 24, 481), leukaemia (Konaka et al., Cell, 1984, 37, 1035) and ovarian, bronchial or pancreatic cancer (EP-A-0400586). As further human tumour tissues are tested for the EGF family of receptor tyrosine kinases it is expected that their widespread prevalence will be established
25 in further cancers such as thyroid and uterine cancer. It has been shown more recently
(W J Gullick, Brit. Med. Bull., 1991, 47, 87) that EGF receptors which possess tyrosine kinase activity are overexpressed in many human cancers such as brain, lung
30 squamous cell, bladder, gastric, breast, head and neck, oesophageal, gynaecological and thyroid tumours.

Accordingly it has been recognised that an inhibitor of receptor tyrosine kinases should be of value as a selective inhibitor of the growth of epithelial cancer cells (Yaish et
35 al. Science, 1988, 242, 933).

It is known from EP-A-0566226 and International Patent
Applications WO-A-96/33980 and WO-A-97/30034 that certain
quinazoline derivatives which possess an anilino substituent at
the 4-position possess EGFR tyrosine kinase inhibitory activity
5 and are inhibitors of the proliferation of cancer tissue.

It is further known from International Patent Applications
WO-A-96/30347 and WO-A-97/38983 that certain structurally-
related quinazoline derivatives possessing an anilino
substituent at the 4-position also possess EGFR tyrosine kinase
10 inhibitory activity.

Subsequently many other 4-anilinoquinazoline derivatives
and structurally-related compounds thereof have been shown to
possess EGFR tyrosine kinase inhibitory activity. Any such
compound possessing that activity and adequate bioavailability
15 is suitable for use in the present invention.

Particular compounds which have been stated to possess
potent EGFR tyrosine kinase inhibitory activity include:-
N-(3-chloro-4-fluorophenyl)-7-methoxy-6-(3-
morpholinopropoxy)quinazolin-4-amine (identified hereinafter by
20 the code number ZD1839);
N-(3-ethynylphenyl)-6,7-bis(2-methoxyethoxy)quinazolin-4-amine
(identified hereinafter by the code number CP358774);
6-acrylamido-N-(3-chloro-4-fluorophenyl)-7-(3-
morpholinopropoxy)quinazolin-4-amine (identified hereinafter by
25 the code number PD0183805) disclosed, for example, in J.Med.
Chem., 1999, 42,1803-1815; and
pyrrolopyrimidine (identified hereinafter by the code number
PKI 166 (CGP75166)).

All of the above relates to treatment of full **cancer**
30 cells. Thus, it was believed that such EGFR tyrosine kinase
inhibitors operate to inhibit the downstream signalling for the
EGFR in ER- cancer cells and thereby inhibit proliferation of
such cells.

As to **normal** cells, it is known that EGF may have a
35 mitogenic effect in a variety of non-transformed epithelial
cells grown in culture (Carpenter et al., Annual Reviews in
Biochemistry, 1979, 48, 193) including cells from mouse mammary

glands but that EGF can also have a growth inhibitory effect in certain cell lines and in naturally proliferating mammary tissue in mice (Coleman et al., Developmental Biology, 1990, 137, 425).

5   We have now found that EGFR tyrosine kinase inhibitors have effects not only on the growth of transformed epithelial cancer cells but also on the constitutive growth of normal epithelial cells. In, for example, normal human mammary epithelial tissue in the woman, oestrogen stimulates normal

10 growth and induces the expression of the progesterone receptor. This allows, the hormonal effects of a second sex steroid to be mediated in the breast epithelium. Constitutive growth of, for example, mammary epithelial cells comprises the non-oestrogen dependent baseline turnover of cells which decreases with the

15 age of the woman and after the menopause.

    Thus using a xenograft model involving the implantation and growth of human normal breast tissue and in situ breast cancer in athymic BALB/c nu/nu mice (Holland et al., J. National Cancer Institute, (1997), 89, 1059 and Gandhi et al,

20 Cancer Research, (2000), 60, 4284 - 4288), we have now found that EGFR tyrosine kinase inhibitors have effects on the constitutive and oestrogen-stimulated proliferation of premenopausal benign and pre-malignant [Ductal Carcinoma In Situ (DCIS)] breast epithelium. The inhibitory effect on

25 constitutive and oestrogen-stimulated growth provides a method for inhibition of the transformation of normal cells into cancerous cells i.e. the basis for the chemopreventative treatment of women, particularly those at higher risk of developing malignant breast cancer.

30   An EGFR tyrosine kinase inhibitor may therefore be used to reduce, preferably to inhibit, the transformation of epithelial cells, in particular mammary epithelial cells, from a normal to a malignant state.

    Given that EGF is known to be able to have a growth

35 inhibiting effect on certain cell lines and naturally proliferating mammary tissue in mice, presumably via its growth receptor, it is surprising that a compound which actually

blocks such growth reception (an EFGR inhibitor) should also reduce proliferation.

Thus, unlike conventional antioestrogens such as Tamoxifen, which effectively operate to block the effect of
5 oestrogen upon the oestrogen receptor and lower EGF/TGFα production by the cell, and contrary to the previously mentioned mechanism of operation of EGFR inhibitors upon ER-cancer cells (again associated with EGFR blockage), it is believed that the EGFR inhibitors, when administered to in vivo
10 benign mammary epithelium or to in vivo non-invasive mammary cancer (whether ER+ or ER-), are operative by blocking directly the cell proliferating role of the tyrosine kinase. Thus, by directly blocking the EGFR tyrosine kinase, the nature of the hormone sensitivity of the cells becomes irrelevant;
15 i.e., the drugs operate by a non-hormonal mechanism.

In more detail, referring firstly to cell growth, in the breast, most growth cells are EGFR+ and ER-. In the absence of an EGFR inhibitor, oestrogen stimulates (only) ER+ cells, which secrete EGF production, which in turn acts on the ER- cells in
20 a paracrine manner to induce proliferation. EGFR TK inhibitors of the invention bind to EGFR directly on ER- cells to inhibit such proliferation.

On the other hand, cell death (apoptosis) is prevented by survival signals from both tissue matrix and growth factors.
25 Insulin like growth factor (IGFI) signals through the IGFI Receptor to prevent cell death in the breast.

Recent work by Roudabush et al (J. Biol. Chem, (2000), 275, 22583 - 22589) has demonstrated that the IGFR stimulation induced by IGFRs leads to secretion of an EGF family member
30 outside the cell which then acts on the cell's own, and neighbouring cells', EGFR. Blockade of the EGFR in the breast by EGFR TK inhibitors in the invention therefore leads to cell death (apoptosis) in vitro and in vivo in breast epithelium.

Surprisingly, the EGFR TKI can act directly, not only on
35 the EGFR expressed on the ER- cells but independently upon ER+ cells.

R B Clarke et al, in Cancer Research (1997 $\underline{57}$ 4987-4991)
have shown that normal ER+/EGFR- epithelial breast cells do not
proliferate whilst neighbouring ER-/EGFR+ cells do, indicating
that proliferation occurs by two mechanisms, namely oestrogen
5 stimulation of

    (1)   ER+ cells and

    (2)   EGF and related ligands secreted by ER+ cells so as
          paracrinely to stimulate neighbouring EGFR+/ER-
          cells.

10   By inhibiting TK with EGFR TKI, neighbouring ER-/EGFR+
cells are prevented from proliferating.

In addition, our data demonstrates that the EGFR TKI also
prevents expression of the oestrogen induced progesterone
receptor in ER+ cells and reduces ER+ cell turnover.

15   Thus, oestrogen induces steroid hormone receptor and
protein synthesis in normal ER+ cells to enable proliferation
and growth and hormone responsiveness of a cell and to secrete
growth stimulating factors (e.g. EGF) which act on neighbouring
ER- breast epithelial cells.

20   Additionally, EGFR TK inhibitors prevent the induction of
the progesterone receptor by oestrogen in ER+ cells, thus
reducing hormonal sensitivity and stimulation of breast
epithelium and preventing transformation of normal to cancer
tissue.

25   The progesterone receptor labelling index (PRLI), which
falls in normal breast tissue unexposed to pre-menopausal
levels of oestrogen to around 5%, is increased on exposure to
oestrogen to 15% (I J Laidlaw et al, Endocrinology (1994), $\underline{136}$,
164-171). The use of ZD 1839 to antagonise oestrogen
30 stimulation abrogates this rise leading to a PRLI of 10%.

In addition, an EGFR tyrosine kinase inhibitor may be used
on epithelial tissue, in particular mammary epithelial tissue,
which has developed to a non-invasive intermediate stage
between normal epithelium, in particular normal breast
35 epithelium, and malignant invasive epithelium, in particular
malignant invasive breast epithelium, either to prevent

proliferation of such cells or to cause the epithelial tissue
in such cells substantially to revert back to a normal state.

Examples of such intermediate stage cells are those
associated with atypical ductal hyperplasia, lobular neoplasia
5 and non-invasive in-situ cancer cells present within the duct
and/or lumen of a breast. Such cells may be present in
patients at high risk of breast cancer.

According to a first aspect of the present invention
there is provided the use of an EGFR tyrosine kinase inhibitor
10 in the manufacture of a medicament for use in reducing,
preferably inhibiting, the transformation of epithelial cells,
in particular mammary epithelial cells, from a normal to a
malignant state in an invasive breast cancer free human,
especially a woman.

15 According to a second aspect of the present invention
there is provided the use of an EGFR tyrosine kinase inhibitor
in the manufacture of a medicament for use in reducing,
preferably inhibiting, the transformation of epithelial cells,
in particular mammary epithelial cells, from an intermediate
20 state, as defined above, to a malignant state in an invasive
breast cancer free human, especially a woman.

The invention, according to each of these first and second
aspects, is particularly applicable to patients at high risk of
breast cancer, so-called "high risk" patients. It is known to
25 classify high risk patients as being those having a score of at
least 1.5 on the so-called Gail Risk Model, a computer
generated programme acceptable for assessment of the risk of
breast cancer (see M. H. Gail et al, J. Natl. Cancer Inst,..,
(1989), 81, 1879 - 1886). Factors which increase the risk of
30 developing breast cancer include the presence of intermediate
cells associated with atypical hyperplasia (including a history
of this as indeed of breast cancer in a first relative),
lobular neoplasia, non-invasive in-situ cancer or the presence
of a mutant BRCAI gene (see D. L. Page et al, BMJ, (1994), 309,
35 61 - 64).

Thus, the invention is especially applicable to the
treatment of intermediate cells.

In a preferred version of the above mentioned first aspect of the invention there is provided the use of an EGFR tyrosine kinase inhibitor selected from ZD1839, CP358774, PD0183805 and PKI 166 in the manufacture of a medicament for
5 use in inhibiting the transformation of mammary epithelial cells from a normal to a malignant state in a human, especially a woman.

In a more preferred version of this aspect of the invention there is provided the use of the EGFR tyrosine kinase
10 inhibitor ZD1839 in the manufacture of a medicament for use in inhibiting the transformation of mammary epithelial cells from a normal to a malignant state in a human, especially a woman.

In a preferred version of the above mentioned second aspect of the invention there is provided the use of an EGFR
15 tyrosine kinase inhibitor selected from ZD1839, CP358774, PD0183805 and PKI 166 in the manufacture of a medicament for use in inhibiting the transformation of mammary epithelial cells from an intermediate to a malignant state in a human, especially a woman.

20   In a more preferred version of this aspect of the invention there is provided the use of the EGFR tyrosine kinase inhibitor ZD1839 in the manufacture of a medicament for use in inhibiting the transformation of mammary epithelial cells from an intermediate to a malignant state in a human, especially a
25 woman.

According to a third aspect of the present invention there is provided a method for reducing, preferably inhibiting, the transformation of epithelial cells, in particular mammary epithelial cells, from a normal to a malignant state in an
30 invasive breast cancer free human, especially a woman, which comprises the administration of an effective amount of an EGFR tyrosine kinase inhibitor.

In a preferred version of this third aspect of the invention there is provided a method for inhibiting the
35 transformation of mammary epithelial cells from a normal to a malignant state in a human, especially a woman, which comprises the administration of an effective amount of an EGFR tyrosine

kinase inhibitor selected from ZD1839, CP358774, PD0183805 and PKI 166.

In a more preferred version of this aspect of the invention there is provided a method for inhibiting the
5 transformation of mammary epithelial cells from a normal to a malignant state in a human, especially a woman, which comprises the administration of an effective amount of the EGFR tyrosine kinase inhibitor ZD1839.

According to a fourth aspect of the present invention
10 there is provided a method for reducing, preferably inhibiting, the transformation of epithelial cells, in particular mammary epithelial cells, from an intermediate to a malignant state in an invasive breast cancer free human, especially a woman, which comprises the administration of an effective amount of an EGFR
15 tyrosine kinase inhibitor.

In a preferred version of this fourth aspect of the invention there is provided a method for inhibiting the transformation of mammary epithelial cells from an intermediate to a malignant state in a human, especially a woman which
20 comprises the administration of an effective amount of an EGFR tyrosine kinase inhibitor selected from ZD1839, CP358774, PD0183805 and PKI 166.

In a more preferred version of this aspect of the invention there is provided a method for inhibiting the
25 transformation of mammary epithelial cells from an intermediate to a malignant state in a human, especially a woman, which comprises the administration of an effective amount of the EGFR tyrosine kinase inhibitor ZD1839.

As in the case of the first and second aspects of the
30 invention, these third and fourth aspects of the invention are particularly applicable to high risk patients and to treatment of intermediate cells, as hereinbefore defined.

According to a fifth aspect of the present invention there is provided the use of an EGFR tyrosine kinase inhibitor in the
35 manufacture of a medicament for use in causing substantial reversion of epithelial tissue, in particular mammary epithelial tissue, back to a normal state from the

intermediate state, as previously defined, between normal
epithelium, in particular normal breast epithelium, and
malignant invasive epithelium, in particular malignant invasive
breast epithelium.

5   In a preferred version of this fifth aspect of the
invention there is provided the use of an EGFR tyrosine kinase
inhibitor selected from ZD1839, CP358774, PD0183805 and PKI 166
in the manufacture of a medicament for use in causing
substantial reversion of mammary epithelial tissue back to a
10 normal state from an intermediate state between normal breast
epithelium and malignant invasive breast epithelium.

        In a more preferred version of this fifth aspect of the
invention there is provided the use of the EGFR tyrosine kinase
inhibitor ZD1839 in the manufacture of a medicament for use in
15 causing substantial reversion of mammary epithelial tissue back
to a normal state from an intermediate state between normal
breast epithelium and malignant invasive breast epithelium.

        According to a sixth aspect of the present invention there
is provided a method for causing substantial reversion of
20 epithelial tissue, in particular mammary epithelial tissue,
back to a normal state from an intermediate state between
normal epithelium, in particular normal breast epithelium, and
malignant invasive epithelium, in particular malignant invasive
breast epithelium which comprises the administration to an
25 invasive breast cancer free human, especially a woman, of an
effective amount of an EGFR tyrosine kinase inhibitor.

        In a preferred version of this sixth aspect of the
invention there is provided a method for causing substantial
reversion of mammary epithelial tissue back to a normal state
30 from an intermediate state between normal breast epithelium and
malignant invasive breast epithelium which comprises the
administration to a human, especially a woman, of an effective
amount of an EGFR tyrosine kinase inhibitor selected from
ZD1839, CP358774, PD0183805 and PKI 166.

35   In a more preferred version of this sixth aspect of the
invention there is provided a method for causing substantial
reversion of mammary epithelial tissue back to a normal state

from an intermediate state between normal breast epithelium and malignant invasive breast epithelium which comprises the administration to a human, especially a woman, of an effective amount of the EGFR tyrosine kinase inhibitor ZD1839.

In use, the EGFR tyrosine kinase inhibitor may be administered alone or in a composition containing a pharmaceutically acceptable excipient. Preferably, it is administered in tablet form.

It will generally be administered so that an effective, non-toxic dose is given. The size of the dose will naturally vary according to the particular EGFR tyrosine kinase inhibitor which is chosen and the route of administration to the patient. In general conventional doses of each EGFR tyrosine kinase inhibitor can be employed. In particular, for the EGFR tyrosine kinase inhibitors CP358774, PD0183805 and PKI 166, conventional doses according to publications concerning these compounds can be employed. More particularly, for the EGFR tyrosine kinase inhibitor ZD1839, a daily dose is administered in the range, for example, from about 10 mg to 5 g, preferably from about 10 mg to 1000 mg, more preferably from about 10 mg to 500 mg (i.e. about 0.2 mg/kg to 100 mg/kg body weight, preferably from about 0.2 mg/kg to 20 mg/kg body weight, more preferably from about 0.2 mg/kg to 10 mg/kg body weight), given if required in divided doses.

The inhibition of cellular transformation defined hereinbefore may be applied as a sole therapy or may involve, in addition to an EGFR tyrosine kinase inhibitor such as ZD1839, one or more other substances and/or treatments. Such conjoint treatment may be achieved by way of the simultaneous, sequential, separate or intermittent administration of the individual components of the treatment. For example, in women at above average risk of developing breast cancer, when the EGFR tyrosine kinase inhibitor is used to inhibit the transformation of mammary tissue into breast cancer, it may be normal practice to use a combination of different forms of treatment. The other component of such conjoint treatment may include an antioestrogen such as tamoxifen, fulvestrant (ICI

182,780: faslodex) or raloxifene. It may then be beneficial to employ sequential therapy with, for example, a first treatment period of about 1 to 6 months during which a conventional dose of an EGFR tyrosine kinase inhibitor such as ZD1839 is

5 administered followed by a second treatment period of about 1 to 6 months during which a conventional dose of an antioestrogen such as tamoxifen, fulvestrant or raloxifene is administered. Thereby a period is allowed whereby some constitutive growth of mammary tissue is permitted in order to

10 minimise the extent of tissue atrophy.

Alternatively the combination therapy may include the continuous administration of an antioestrogen such as tamoxifen, fulvestrant or raloxifene and the intermittent administration of an EGFR tyrosine kinase inhibitor such as

15 ZD1839. The intermittent therapy of the EGFR tyrosine kinase inhibitor may involve, for example, a two-monthly cycle of treatment comprising a first portion involving the dosing of the EGFR tyrosine kinase inhibitor such as ZD1839 for a period of about one month followed by a second portion involving an

20 EGFR tyrosine kinase drug-free period of about one month. Thereafter further two-monthly cycles of such treatment may be given.

Embodiments of the invention will now be described in more detail with reference to the following Examples and

25 accompanying drawings in which:

Fig. 1 illustrates, for Example 2, proliferation (Ki67 LI) and apoptosis (AI) in ER-/EGFR+ and ER+/EGFR- DCIS epithelium; and

Fig. 2 illustrates, for Example 3, ZD18389 dose response

30 in (a) DCIS and (b) normal breast.

Example 1

The effect of the EGFR tyrosine kinase inhibitors of the present invention was determined using a xenograft model involving the implantation and growth of human normal breast tissue and in situ breast cancer in female athymic BALB/c nu/nu mice (Holland et al., J. National Cancer Institute, 1997, 89, 1059 and Gandhi et al, Cancer Research, (2000), 60, 4284 - 4268). Normal breast tissue and ductal carcinoma in situ (DCIS) tissue from women undergoing therapeutic surgery was implanted as xenografts into female mice. After 14 days, daily dosing of ZD1839 at 10 to 200 mg/kg was commenced for a 14 day period. Xenograft tissue was excised at days 0, 14, 21 and 28. Pellets of 17β-estradiol (2mg) were implanted subcutaneously in a second set of experiments (using normal breast xenografts) on day 14 in both control and treatment mice and conventional Ki67 immunostaining was used to assess epithelial proliferation (Ki67 is a monoclonal antibody to a marker in a cell).

The following results were obtained :-

Ki67 index	Day 0	Day 21		Day 28	
Normal breast	Control	Control	ZD1839 Group	Control	ZD1839 Group
Median	7.6	3.3	0.7	6.1	1.8

Ki67 index	Day 0	Day 21		Day 28	
DCIS tissue	Control	Control	ZD1839 Group	Control	ZD1839 Group
Median	19.9	10.6	7.2	23	3.2

Ki67 index	Day 0	Day 21		Day 28	
Oestrogen-stimulated Normal breast	Control	Control	ZD1839 Group	Control	ZD1839 Group
Median	10.4	15.7	1.5	14.8	1.2

Example 2

Summary of Procedure

5 Breast tissue from 16 women undergoing surgery for DCIS were implanted into 16-20 immunosupressed mice per experiment (8 xenografts/mouse). Treatment commenced 2 weeks post implant and consisted of daily gavage with ZD1839 at 10-200mg/Kg for 14-28 days; appropriate controls were present. Xenografts were
10 removed on Days 14, 21, 28 and 42 and then assessed for proliferation (LI) by Ki67 immunostaining, and apoptosis (AI) by morphology.

Materials and Methods
Patients

Participants in this study were women who either attended the Nightingale

Breast Screening Assessment Centre or the Symptomatic Breast Clinic at the

University Hospital of South Manchester, U.K. during the period November

1998 to January 2000. Women were included in the study if they had

mammograms showing widespread microcalcification indicative of DCIS and

either cytopathological or histopathological confirmation of the diagnosis

(n=16). All tissue samples were obtained at therapeutic excision of DCIS.

Approval to remove tissue from pathologic samples in this study was granted

by the South Manchester Medical Research Ethics Committee.

Animals

Intact, adult, female, 8-10 week-old, athymic nude mice (BALB/c nu/nu) were

obtained from the breeding colony at the Paterson Institute for Cancer

Research. They were housed under conventional conditions with a 12-hour

cycle of light and dark (lights off from 7 PM to 7 AM) in filter top cages. They

were supplied ad libitum with irradiated feed and filtered water and irradiated

bedding during breeding. Normal food, water, and bedding were used during

the experiments. All care of the animals and surgical procedures were

performed in accordance with Home Office Regulations and the UK Scientific

Procedures (1986) Act. Halothane inhalational anaesthesia (2-4% halothane

on oxygen; Halovet Vapouriser, International Market Supplies, Congleton,

U.K.) was used for all procedures.

Treatment of Tissue Samples

For preparation of tissue specimens for grafting to mice, 1-2 cm^3 pieces of

breast tissue, containing microcalcifications, were taken at the time of surgery

from the main specimen. The fresh tissue was stripped of excess fat and

immediately divided under sterile conditions into three or four smaller portions

and placed in Dulbecco's Modified Eagle Medium (DMEM), with 4.5 g/L

glucose and without sodium pyruvate (Gibco Life Technology, Paisley,

Scotland, UK) at room temperature until implantation into the mice. In the

laboratory, the tissue was placed into fresh DMEM in a sterile Petri dish, and

the tissue was carefully dissected into 2-mm x 2-mm x 1-mm samples with a

scalpel blade. Depending on the volume of tissue available, between 5 and 20

pieces were randomly selected from the Petri dish and not implanted into the

mice but instead were used for histology. Half of these nonimplanted (day 0)

grafts were immediately fixed in buffered formalin (4% formaldehyde) for 24

hours, and the other half in Carnoy's fixative for 1 hour, and then all were

stored in 70 % alcohol. Following at least 24 hours, the fixed tissue samples

were placed individually in tissue cassettes (Tissue Tek III; Bayer Diagnostics

Ltd., Basingstoke, UK.) and stored in 70% alcohol until paraffin embedding.

These samples representing the DCIS excised from the each patient, were

labelled as the "Day O" specimens, and were reserved for histological review,

immunostaining, and apoptotic cell counts. The remaining xenografts were

implanted into nude mice.

Implantation of Xenografts into nude mice

As in Example 1,   each patient's sample was divided between 10

and 32 mice (depending on the volume of tissue and number of mice

available; median number, sixteen). Transplantation of xenografts onto the

mice was completed within 90 minutes of removal of tissue from the patient.

Two small midline skin incisions were made across the dorsal skin through

which 8 tissue pieces were symmetrically placed (4 on each side).

Retrieval of these xenografts at the appropriate time points required

reanaesthetizing the mice and excising each graft using sharp dissection. The

grafts were then processed for histology as described above for the Day 0

specimens. For the 100-200 mg/kg ZD1839 experiments, grafts were

removed on Days 14, 21 and 28; at the lower doses (10-75
mg/kg), grafts were retrieved on Days 14, 28, and 42. Two
xenografts were removed at each interim time point and 4
xenografts at each end time points from each mouse.

Treatment

The mice were gavaged for 14-28 days with either ZD1839 (10-200mg/kg) or

vehicle commencing on Day 14 after removal of the first 2 xenografts. ZD1839

(4-(3chloro-4flurophenylamine)-7-methoxy-6(3-(4morpholinyl)quinazoline), an

orally active, selective EGFR-TKI was a kind gift of AstraZeneca

Pharmaceuticals. The vehicle (control) was 0.5% polysorbate.

Histological Evaluation of Xenografts

All Day 0 specimens and each xenograft were embedded into paraffin blocks.

H&E stained, 3-μm sections from each block were examined by a single

experienced breast pathologist   for the presence of DCIS   ; those

.containing DCIS were assessed for apoptosis and Ki67 antigen

immunogenicity (as a marker of epithelial proliferation) as previously

described.

Nuclear grade and the presence or absence of comedo-necrosis were also

ascertained on all Day O specimens containing DCIS.   In addition, Day

0 specimens were evaluated immunohistochemically for ER, c-erbB-2, EGFR

status.

Assessment of Apoptotic cell death

H&E-stained sections of DCIS samples were examined using light

microscopy for morphological evidence of apoptosis. The criteria used to

identify apoptotic cells are well recognised, and include condensation of

chromatin initially at the margins of the nucleus; condensation of the

cytoplasm (chromophilia); detachment from the surrounding cells, indicated by

the appearance of a characteristic white halo around the dying cell; and

cytoplasmic budding to form membrane-bound fragments (apoptotic bodies).

To acquire the AI, at least 1000 cells were counted at x400 magnification

using a Zeiss microscope, and the number of cells displaying apoptotic

morphology were expressed as a percentage of the total number counted.

Immunohistochemical determination of ER and Ki67 Nuclear Antigen

ER and Ki67 immunohistochemical methodology were employed. These have been

previously described (Gandhi et al, Br. J. Cancer, (1998), 78, 785-794).

Both ER and Ki67 staining were predominantly nuclear with minimal

cytoplasmic uptake. The intensity of staining was variable, but this was not

assessed separately, and the cells were judged as positive or negative.

The Ki67 the Labelling Index (LI) was calculated from counting a minimum of

1000 epithelial cells and the number of positively stained nuclei were

expressed as a percentage of the total number counted. The ER status was

determined also counting at 1000 cells, and lesions were considered ER+ if >5% of cells were positively stained for ER

Immunohistochemical determination of c-erbB-2, EGFR and pErk1/Erk2

Paraffin wax sections (3μm thick) of tissue from each specimen were cut, mounted on APES (3-aminopropylethoxysilane) coated slides, dewaxed and hydrated before immunohistochemical staining for the c-erbB-2, EGFR and phosphorylated (p) Erk1/Erk2. Antigen retrieval was achieved by a microwave method (650W) for 30 minutes (min). Endogenous peroxidase activity was blocked by incubation in hydrogen peroxide [1.5% (v/v) for EGFR and c-erbB-2, 3% (v/v) for pErk1/Erk2] in phosphate-buffered saline (PBS) for 15 min. For the c-erbB-2 and EGFR antigen, the slides were rinsed in TBS prior to using 10% normal rabbit serum to block non-specific binding and then incubated with the primary antibody at a dilution of 1:20 for EGFR (mouse monoclonal anti-human, NCL-EGFR; Novocastra labs, Newcastle upon Tyne, UK) and at a dilution of 1:40 for c-erbB-2 (mouse monoclonal, MAB4022, Chemicon, Harrow, UK), for 1 hour at room temperature. A biotinylated rabbit anti-mouse inmmunoglobulin (E413, Dako, High Wycombe, UK) was applied as the secondary antibody (1:350 dilution, incubated for 30 min), and following this a 30 min incubation at RT with the standard three layered streptavidin-avidin-biotin horseradish (K0377, Dako) for c-erbB-2 and EGFR.

For pErk1/Erk2 antigen detection, the sections were blocked with 20% normal human serum for 15 min prior to application of the polyclonal rabbit antihuman pMEK, pErk1/Erk2, pElk1 primary antibodies at a dilution of 1:20 (9101L, New England Biolabs, UK). Biogenex Multil Link at a dilution of 1:100 (Euro/DPC, Llanberis, UK) was applied as the secondary antibody for 1 hr. An avidin/biotin

complex immunohistochemical kit procedure (Biogenex Concentrated Label,

Euro/DPC) was employed.

The chromogen diaminobenzidine (Sigma, Poole, UK) was applied for 5 min

and then haematoxylin as counterstain for 5 min.

Negative (generic mouse IgG, X0931,Dako) and positive controls (sections of

A431 cells for EGFR, and of DCIS tissue previously shown to be strongly

positive for c-erbB-2 and pErk1/Erk2) were used.

Staining for c-erbB-2 and EGFR were predominantly membranous but there

was also cytoplasmic and nuclear uptake. These were scored positive (scale

1-4) or negative in relation to the positive and negative control slides. Staining

for pErk1/Erk2 was nuclear and cytoplasmic and scored according intensity of

staining in the nuclear and cytoplasmic components as previously

described (Gee et al, Int. J. Cancer, (1995), 64, 269 - 273).

All histological assessments were performed by investigators blinded to the

treatment group. Reproducibility of counting was assessed by the same

investigator re-scoring 10 slides stained with the Ki67 antibody and 10 H&E

slides for apoptosis several months after initial estimation. The two sets of

results thus obtained were well correlated by regression analysis (r=0.95,

P<0.001)

Epithelial proliferation in normal mouse intestine

Removal, processing of intestinal tissue for Ki67 assessment, and scoring methodology has

been previously described (Potten and Grant, Br. J. Cancer, (1998), 78, 993-1003).

Statistical Methods

Statistical analysis was performed using SPSS software (SPSS, Chicago, IL,

USA) by the CRC Department of Computing and Biomathematics at the

Paterson Institute for Cancer Research. For each case showing normal breast epithelium or DCIS, a comparison was made between samples retrieved from ZD1839 treated and control mice at each assessment time point. The tissue samples obtained at each assessment time in the two study groups were considered to be statistically independent and were compared using the Mann Whitney U test. All significance tests were two-sided, using the conventional 5% significance level. Data from the separate ZD1839 doses were collapsed after initial analysis demonstrated effect at all doses. Pearson's correlation coefficient was used to examine the degree of correlation between metric variables. Statistical analysis of proliferation in intestinal epithelium was by comparing medians at each epithelial cell position in the crypts.

Results

The median age of women who were undergoing surgery was n=range (menopausal status).

Xenograft implant and retrieval

DCIS

Of the 16 breast tissue specimens found to contain DCIS at surgery, 13 (81.3%) produced Day 0 specimens which contained DCIS, of which 11 immunohistochemically expressed EGFR+ with a median score of 2 (range 1-3), ᵢ and 2 were EGFR-. Of these 11 EGFR+ cases, 7 were ER-/c-erbB-2 positive (all high nuclear grade), and 3 ER+/c-erbB-2 positive (1 high grade, 2 intermediate grade), and 1 ER+/c-erbB-2 negative (low grade).

The 13 breast tissue specimens gave rise to a total of 273 Day 0 samples of which 44 (16.1%) contained foci of DCIS. A total of 1904 xenografts were implanted, of which 1858 (97.5%) were successfully retrieved. Of the 1858 xenografts retrieved, 329 (17.7%) contained DCIS. The median retrieval of DCIS per experiment was 71% of that expected (range 14-91.3%). DCIS was retrieved at Days 14, 21, 28, and 42 xenografts in all of the 13 experiments.

Normal breast

Histologically normal breast tissue was found in all 16 specimens removed from surgery for DCIS. The majority (97.6%) of the xenografts implanted were retrieved, of which 22.4% contained normal breast. Normal breast was retrieved from all the time points in 100% of all of the experiments.

All 16 cases were ER+ and EGFR+ with a median score of 1 (range 1-2), and 2 were also weakly c-erbB-2 positive.

Normal Breast (Table 1)

Table 1 shows EGFR+ proliferation (LI) and apoptosis (AI) in ZD1839 (10-200 mg/kg [collapsed data]) versus vehicle-treated (a) normal breast and (b) DCIS epithelium at the different time points. Numbers in parentheses represent the interquartile ranges **$p<0.01$, ***$p<0.001$ versus control.

Normal breast xenografts had a decrease in median LI from Days 0 to 14 (4.9 [IQR: 4.0-5.7] versus 3.8 [IQR: 2.7-5.1], $p<0.05$) which then remained unchanged for the other time points (Days 21, 28, and 42). Median LI decreased in the ZD1839 treated group compared to controls at Day 21 (2.3 [IQR: 1.0-3.8] versus 4.1 [IQR: 2.8-5.2]), Day 28 (2.2 [IQR: 1.7-3.3] versus 4.1 [IQR: 2.4-5.4]), and Day 42 (1.7 [IQR: 1.1-2.6] versus 2.8 [IQR: 2.3-5.1]) (all $p<0.01$).

The AI findings were similar findings to the DCIS treated xenografts above. No reduction in median AI was observed from Day 0 to 14 (0.20 [IQR: 0.17-0.29] versus 0.18 [IQR: 0.10-0.21], $p=0.90$). At Day 21, there was an increase in median AI in ZD1839 treated xenografts compared to controls (0.36 [IQR: 0.23-0.53] versus 0.18 [IQR: 0.10 to 0.25, $p<0.001$). After 14 days of treatment (Day 28), median AI equilibrated to control levels (ZD1839 0.19 [IQR: 0.10-0.28] versus Control 0.18 [IQR: 0.10-0.20], $p=0.35$).

Table 1

The effect of ZD1839 on proliferation and apoptosis on normal breast and DCIS epithellum

Median	Day 0	Day 14	Day 21 Control	Day 21 ZD1839	Day 28 Control	Day 28 ZD1839
LI						
Normal breast	4.9 (4.0-5.7)	3.8 (2.7-5.1)	4.1 (2.8-5.3)	2.3** (1.0-3.8)	3.4 (2.4-5.4)	2.2** (1.7-3.3)
DCIS	13.9 (11.9-15)	12.6 (9.1-14.1)	11.2 (9.7-12.9)	4.6** (3.2-7.2)	13.1 (11.8-15.8)	5.1** (3.2-10.6)
AI						
Normal breast	0.20 (0.18-0.29)	0.18 (0.10-0.21)	0.18 (0.10-0.25)	0.36** (0.23-0.53)	0.18 (0.10-0.20)	0.19 (0.10-0.28)
DCIS	1.07 (0.89-1.21)	0.79 (0.53-1.19)	0.72 (0.55-0.91)	1.47** (1.25-2.18)	0.46 (0.34-0.85)	0.64 (0.38-1.15)
LI/AI						
Normal breast	23.9 (18.3-43.3)	25.7 (13.4-33.3)	15.9 (8.0-26.3)	3.9*** (1.6-5.2)	33.8 (21.4-70.6)	8.5*** (5.8-17.4)
DCIS	12.1 (8.4-15.8)	12.1 (8.2-19.9)	17.7 (12.7-22.0)	2.7*** (2.2-3.6)	29.7 (13.1-40.2)	4.4*** (2.7-6.9)

EGFR+ DCIS (Fig. 1)

Figure 1 shows changes in LI and AI induced by ZD1839 in
ER-/EGFR+ (1a and 1c) and ER+/EGFR+ (1b and 1d) DCIS
5 respectively. ZD1839 or vehicle was given from day 14 onwards.
Values for days 0 and 14 are from untreated mice. An increase
in AI and a decrease in LI was seen in the ZD1839 group
following 7 and 14 days of treatment (*p<0.05, **p<0.01 versus
control) in both ER-/EGFR+ and ER+/EGFR+ DCIS. Numbers in
10 parentheses represent the number of observations seen for that
particular time point and for that group. Median values are
shown as thick horizontal lines, boxes represent interquartile
range.

The median LI and AI in Day 0 specimens was higher in the 7 cases of ER-
15
/EGFR+ DCIS than the 4 cases of ER+/EGFR- DCIS (14.0 [IQR:13.0-15.3]

versus 7.8 [IQR:6.5-11.2], and 1.3 [IQR: 1.0-1.6] versus 0.79 [IQR: 0.6-1.0]

respectively, both p<0.05).

20 Median LI in ER-/EGFR+ DCIS decreased in the ZD1839-treated group

compared to the vehicle-treated group by Day 21 (4.7 [IQR: 2.6-17.2] versus

11.6 [IQR: 10.7-15.1], p=0.19), Day 28 (8.3 [IQR: 2.7-10.9] versus 13.5 [IQR:

12.0-16.3], p<0.01), and Day 42 (11.4 [IQR: 10.8-11.8] versus 13.0 [IQR:
25
12.0-14.8], p<0.05) (Fig. 1a).

There was no change in median AI in ER-/EGFR+ DCIS from Day 0 to 14 (1.3

[IQR: 1.0-1.6] versus 1.0 [IQR: 0.8-1.44], p=0.14). Following 7 days of

30 treatment (Day 21), there was a significant increase in median AI in the

ZD1839-treated group compared to the vehicle-treated group (2.1 [IQR: 1.0-

2.3] versus 0.7 [IQR: 0.5 to 1.1], p<0.01), although, after 14 days of treatment

(Day 28), median AI returned to control levels (ZD1839 1.1 [IQR: 0.8-1.2]
35
.versus Control 1.2 [IQR 0.5-1.5], p=0.44).(Fig. 1b).

Similar changes in LI and AI in ER+/EGFR+ DCIS (Fig. 1b and 1d) were seen with a significant fall in LI at Day 14 (ZD1839 4.6 (IQR 3.9-5.2%) versus 11.2 Control (IQR 9.2-15.55) and a rise in apoptosis (ZD1839 1.5 (IQR 1.3-1.6) versus

5 Control (IQR 0.6-0.9%).

K167 LI in EGFR+ (Fig. 2)

Figure 2 shows epithelial proliferation rates (Ki67 LI) in EGFR+ (a) DCIS and (b) normal breast xenografts following 2 weeks (Day 28) of gavage with either different doses of ZD1839

10 (10-200 mg/kg) or vehicle control. Numbers in parentheses represent the number of observations seen for that particular time point and for that group. Median values are shown as thick horizontal lines, boxes represent interquartile range.

Increasing doses of ZD1839 were associated with increasing

15 **falls** in epithelial proliferation in both ER-/EGFR+ and ER+/EGFR+ DCIS but not in normal breast (Fig. 2).

Correlation of Proliferation with Apoptosis

We have previously demonstrated a positive correlation of LI with AI in

DCIS.   LI displayed a positive correlation with AI in the normal breast and

DCIS ($r=0.10$, $p=0.02$, and $r=0.25$, $p<0.01$ respectively) not treated by ZD1839

(i.e.Days 0, Day 14, and vehicle control cases).

Comparison of cell population using the median LI/AI ratio (as a ratio of cell

turnover) revealed that ZD1839 treatment significantly inhibited cell turnover in

both DCIS and normal breast at Day 28 (4.4 [2.7-6.9] versus 29.7 [13.1-40.2],

and 8.5 [5.8-17.4] versus 33.8 [21.4-70.6] respectively, $p<0.001$.

Downregulation of pErk1/2 in EGFR TKI treated DCIS and normal breast

To determine whether the MAP kinase signalling pathway is effected by

EGFR tyrosine kinase inhibition, immunohistochemical detection of

phosphorylated (p) ErK1/Erk2 was performed on Day 0 and Day 42 sections

of DCIS and normal breast xenografts treated with ZD1839 or vehicle.

Median Day 0 DCIS and normal breast nuclear HScore were 30 (IQR: 12-45)

and 22 (IQR: 12-34).   In DCIS, the nuclear HScore was decreased in the

ZD1839 treated group compared to controls after 28 days treatment (8 [IQR:

5-18] versus 25 [IQR: 8-30], $p<0.05$). In normal breast there was a similar

difference (7 [IQR: 3-17.5] versus 18 [IQR: 8-32], $p<0.01$).

The Nuclear HScore of pErK1/Erk2 correlated with the Ki67 LI in DCIS and in

normal breast (both $r=0.52$, $p=0.05$).

Discussion

ER- DCIS overexpresses c-erbB-2 oncogene, and also is reported to express EGFR in 50% of cases. We found dual expression of both receptors on the same cell population in 10 out of 11 (90%) of ER- DCIS. ER- DCIS has a high proliferative rate, which in pre-treatment samples correlated with high expression of the MAP kinase signal transduction enzyme.

We therefore hypothesised that the formation of EGFR/c-erbB-2 heterodimers was responsible for the high proliferation rate and MAP kinase expression and we tested our theory by using an EGFR-TKI peptide, ZD1839. On ZD1839 treatment, a prolonged fall in epithelial proliferation and a decrease in activated MAP kinase expression was seen combined with an increase in apoptosis at an early stage confirming our hypothesis. EGFR is known to produce a mitogenic signal and correlates with proliferation and tumour doubling in invasive breast cancer, and inhibition of this pathway led to a fall in Ki67 labelling index in DCIS xenografts.

An increase in apoptosis was seen in both DCIS and normal breast epithelium after seven days of treatment. Although the Insulin Like Growth Factor-1 (IGF-1) is believed to be important in cell survival, recent work by Roudabush et al, J. Biol. Chem., (2000), 275, 22583-22589, has indicated that IGF-1 receptor stimulation leads to secretion of heparin binding-EGF extracellularly and promotes transactivation in the EGF receptor. Thus, the EGFR may be more important to cell survival in the normal breast than has been previously recognised. Since epithelial proliferation correlates with apoptosis in the normal breast a reduction in proliferation would be expected to correlate with a reduction in apoptosis. After the initial

increase in apoptosis after 7 days in the ZD1839 treated group the fall in

labelling index (proliferation) led to a fall in apoptosis by 14 days. However

the overall LI/AI ratio in the ZD1839 treated groups in both DCIS and normal

breast was reduced indicating a lower overall cell turnover with EGFR-TK

inhibition. Moreover, the overall fall in cell turnover indicates the potential

chemopreventative effect of an EGFR TKI on normal breast.

ER- high grade DCIS is reported to be the most likely to relapse after wide

local excision and at relapse at least 50% has become invasive high grade

breast cancer with associated nodal or distant metastases.   Prevention of

relapse and progression to invasive cancer requires an agent which

suppresses proliferation or increases apoptosis and the EGFR-TKI described

fulfils this criteria.

A   suppression of normal breast epithelial proliferation combined with

an increase in apoptosis indicates the potential of EGFR-TK Inhibitors as

chemoprevention agents in women at increased risk of breast cancer.

The EGFR MAP Kinase pathway is believed to be important in cell

proliferation, survival and differentiation.   The significant positive

correlation of pErk1/Erk2 with Ki67 labelling index in DCIS and normal breast

suggests that MAP kinase signalling is important in mediating cell proliferation

in breast epithelium. A decrease in pErk1/Erk2 in the ZD1839 treated group

correlated with a fall in proliferation and it is likely that the EGFR signals

through this pathway and MAP kinase changes will predict drug response.

Example 3

Using the method of <u>Example</u> 1, the procedural steps of which
are described more fully in Example 2, the effect of ZD 1839 on
proliferation (Ki67 LI) in oestrogen stimulated normal breast
5 epithelium was measured. The results are shown in Table 2.

In the normal breast epithelium, on day 0, the date of
implantation into a mouse, the Ki67 LI value was 4.1 and
reduced to 2.6 on the 14th day. Control mice were then treated
with oestrogen alone, while the test mice were treated with
10 both oestrogen and ZD1839. The amount of oestrogen administered
corresponded to a premenopausal level; i.e. 17-β-oestradiol
pellet (2mg) (see Holland et al, referred to in Example 1).
After 21 days, the Ki67 LI value for the control mice was as
high as 6.5, whereas for the test mice the value was
15 considerably reduced to a value of 1.4. After 28 days, the Ki67
LI value for the control mice was 5.4, while for the test mice,
the value continued to fall, to a level as far as 1.1.

Table 2

The effect of ZD 1839 on proliferation (Ki67 LI) in oestrogen
20 stimulated normal breast epithelium

Median Ki67 Labelling Index	Day 0	Day 14	Day 21		Day 28	
			Control	ZD1839	Control	ZD1839
Estrogen stimulated normal breast	4.1 (3.2-5.3)	2.6 (2.1-4)	6.5 (5.8.6)	1.4*** (0.8-1.9)	5.4 (4.6-6.1)	1.1*** (0.9-1.6)

25 Numbers in parenthesis represent interquartile ranges   *p<0.05, **p<0.01, ***p<0.001 vs control

Example 4

Again using the method of Example 1, the effect of ZD1839 on
apoptosis (AI) in oestrogen stimulated normal breast epithelium
30 was measured. The results are shown in Table 3.

In the normal breast epithelium, on day 0, the date of
implantation into a mouse, the AI value was 0.3 and reduced to
0.2 on the 14th day. Control mice were then treated with
oestrogen alone, while the test mice were treated with both
35 oestrogen and ZD1839. The amount of oestrogen administered
corresponded to a premenopausal level. After 21 days, the AI
value for the control mice was as high as 0.5, whereas for the

test mice the value was even higher, at a value of 0.7. After
28 days, the AI value for the control mice was 0.6, while for
the test mice, the value remained at a level of 0.7.

<div align="center">Table 3</div>

5 The effect of ZD1839 on apoptosis (AI) in oestrogen stimulated
normal breast epithelium

Median Apoptotic Index	Day 0	Day 14	Day 21		Day 28	
			Control	ZD1839	Control	ZD1839
Oestrogen stimulated normal breast	0.3 (0.19-0.3)	0.2 (0.1-0.2)	0.5 (0.45-0.65)	0.7 (0.55-0.78)	0.6 (0.45-0.66)	0.7 (0.57-0.81

Numbers in parenthesis represent interquartile ranges   **p<0.01 vs control

Example 5

Using the same application regime as in Example 1, the effect
15 of ZD1839 on progesterone receptor expression (PR LI) in
oestrogen stimulated normal breast epithelium was measured
(progesterone receptor increases the hormonal responsiveness of
the cell). The results are shown in Table 4.

In the normal breast epithelium, on day 0, the date of
20 implantation into a mouse, the PR LI value was 11.8 and reduced
to 5.8 on the 14th day. Control mice were then treated with
oestrogen alone, while the test mice were treated with both
oestrogen and ZD1839. The amount of oestrogen administered
corresponded to a premenopausal level. After 21 days, the PR LI
25 value for the control mice rose to as high as 17.2, whereas for
the test mice the value was only 10.4. Oestrogen induction of
PR expression was therefore suppressed by ZD1839. Thus, the
hormonal effects (normally mediated through the progesterone
receptors) of the sex steroid progesterone and androgen are
30 inhibited.

Table 4

The effect of ZD1839 on PR expression (PR LI) in oestrogen stimulated normal breast and epithelium

Median PR LI (%)	Day 0	Day 14	Day 21 Control	ZD1839
Oestrogen stimulated normal breast	11.8 (6.9-13.5)	5.8 (4.5-7.7)	17.2 (13.5-22.0)	*10.4 (8.9-13.1)

Numbers in parenthesis represent interquartile ranges   **$p<0.001$ vs control

From the above results, it can be seen that as stated hereinbefore, the inhibitory effect on constitutive growth and promotive effect on the death of normal breast cell tissue provides a method for inhibition of the transformation of normal cells into cancerous cells i.e. the basis for the chemopreventative treatment of women, particularly those at higher risk of developing malignant breast cancer.

Studies of normal breast epithelium show that hormone replacement therapy (HRT) increases expression of progesterone receptor (see Hargreaves et al, Br. J Cancer, (Oct. 1998), 78(7), 945-949 and Hofscth et al, J. Clin. Endocrinol. Metab., (Dec 1999), 84(12), 4559-4565) and combined HRT (oestrogen and progesterone) increases breast epithelial proliferation significantly more than does HRT with oestrogen alone (see Hofstch, Supra). Longer term combined HRT use has an increased breast cancer induction rate as compared with HRT with oestrogen alone (see Persson et al, Cancer Causes Control, (Aug 1999), 10(4), 253-260; Colditz, J. Womens Health, (Apr 1999), 8(3), 347-357; and Magnusson et al, Int. J. Cancer, (5 May 1999), 81(3), 339-344). By inhibiting the induction of progesterone receptor, progesterone cannot exert its effects and the frequency of breast cancer development is reduced.

In addition, the inhibiting effect upon oestrogen stimulated normal breast tissue indicates that EGFR tyrosine kinase inhibitor will work alone even in the presence of high levels of oestrogen: i.e. to provide chemoprevention in premenopausal women.

Thus, EGFR-TK inhibition offers a novel approach to the treatment and prevention of cancer regardless of ER status and provides a potential new chemopreventative approach, especially in high risk breast cancer families.

5

CLAIMS

1.   Use of an EGFR tyrosine kinase inhibitor in the
manufacture of a medicament for use in reducing the
transformation of epithelial cells from a normal to a malignant
5 state in an invasive breast cancer free human.

2.   Use of an EGFR tyrosine kinase inhibitor in the
manufacture of a medicament for use in reducing the
transformation of epithelial cells from an intermediate state,
10 between normal epithelium and malignant invasive epithelium, to
a malignant state in an invasive breast cancer free human.

3.   Use of an EGFR tyrosine kinase inhibitor in the
manufacture of a medicament for use in causing substantial
15 reversion of epithelial tissue back to a normal state from an
intermediate state, between normal epithelium and malignant
invasive epithelium.

4.   Use of an EGFR tyrosine kinase inhibitor in the
20 manufacture of a medicament for use in causing substantial
reversion of epithelial tissue back to a normal state from the
intermediate state between normal epithelium and malignant
invasive epithelium.

25 5.   Use according to any preceding claim, wherein the
epithelial tissue is mammary epithelial tissue.

6.   Use according to any preceding claim, wherein the EGFR
tyrosine kinase inhibitor is selected from ZD1839, CP358774,
30 PD0183805 and PKI 166.

7.   Use according to claim 6, wherein the EGFR tyrosine kinase
inhibitor is ZD1839.

35 8.   A method for reducing the transformation of epithelial
cells from a normal to a malignant state in an invasive breast

cancer free human, which comprises the administration of an
effective amount of an EGFR tyrosine kinase inhibitor.

9.   A method for reducing the transformation of epithelial
5 cells from an intermediate to a malignant state in an invasive
breast cancer free human, which comprises the administration of
an effective amount of an EGFR tyrosine kinase inhibitor.

10.   A method for causing substantial reversion of epithelial
10 tissue back to a normal state from an intermediate state,
between normal epithelium and malignant invasive epithelium,
which comprises the administration to an invasive breast cancer
free human, of an effective amount of an EGFR tyrosine kinase
inhibitor.
15

11.   A method according to claim 10, wherein the epithelial
tissue is mammary epithelial tissue.

12.   A method according to any one of claims 8 to 11, wherein
20 the EGFR tyrosine kinase inhibitor is selected from ZD1839,
CP358774, PD0183805 and PKI 166.

13.   A method according to claim 12, wherein the EGFR tyrosine
kinase inhibitor is ZD1839.
25

Figure 1

Proliferation (Ki67 LI) and apoptosis (AI) in ER-/EGFR+ and ER+/EGFR+
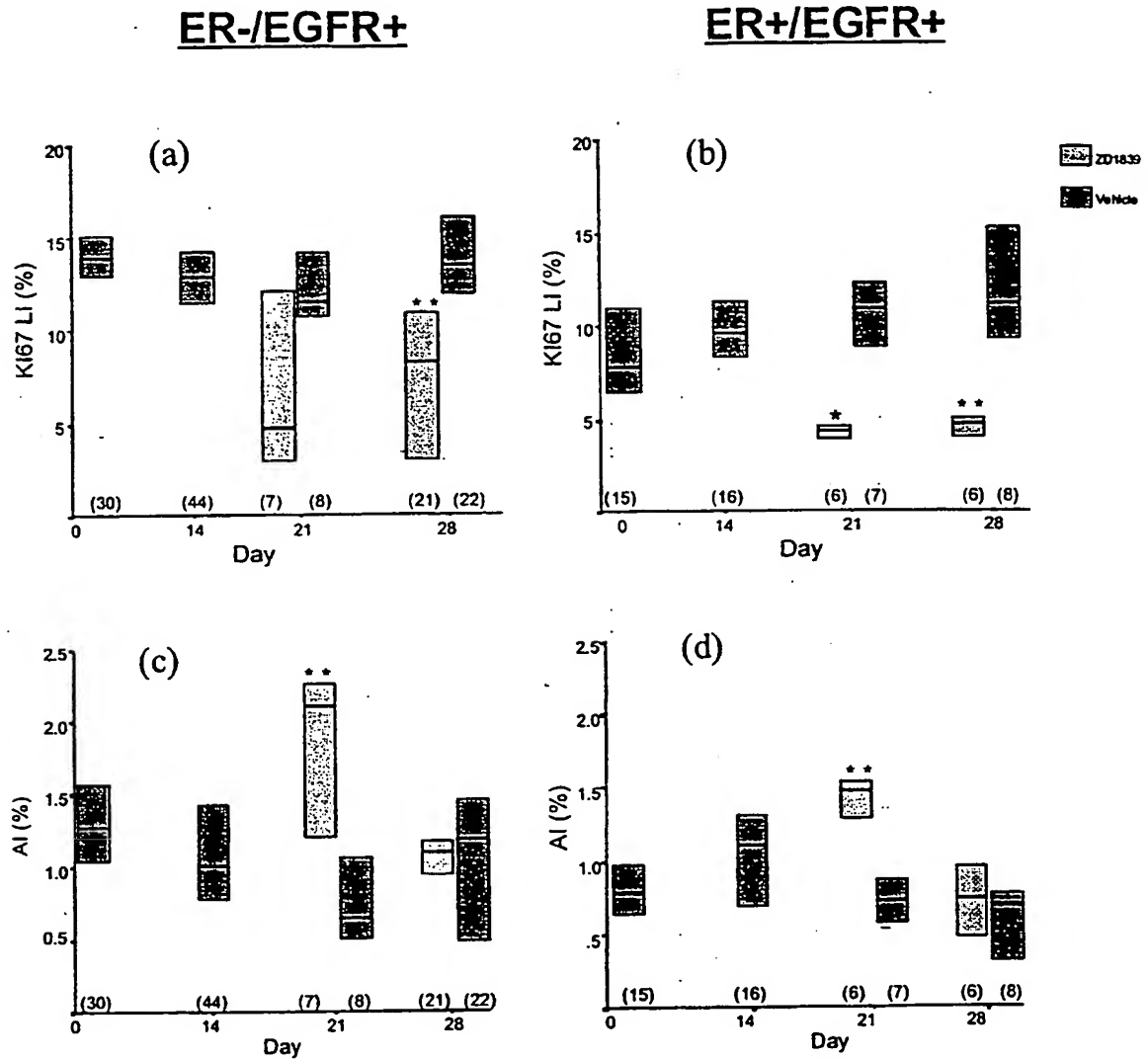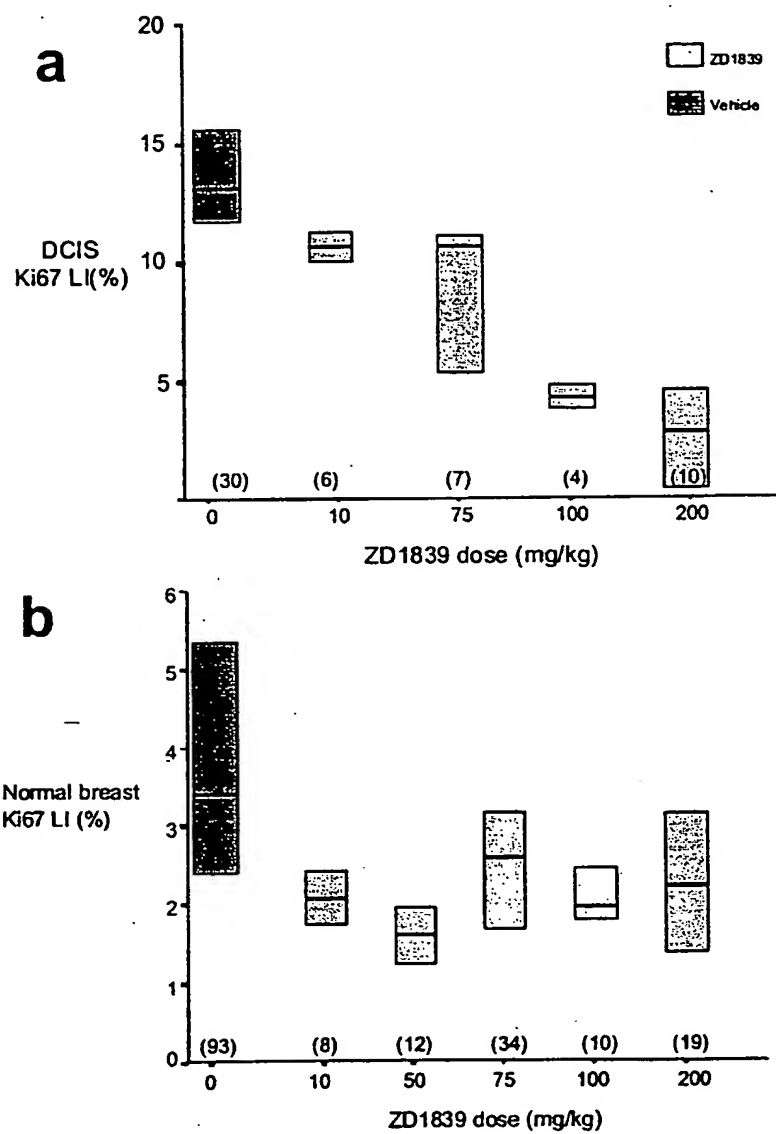
DCIS epithelium.

Figure 2

ZD1839 dose response in (a) DCIS and (b) normal breast

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: USE OF EGFR TYROSINE KINASE INHIBITORS FOR TREATING BREAST CANCER

(57) Abstract: An EGFR tyrosine kinase inhibitor is used in the manufacture of a medicament for use in (a) reducing the transformation of epithelial cells from a normal to a malignant state in an invasive breast cancer free human; and/or (b) reducing the transformation of epithelial cells from an intermediate state, between normal epithelium and malignant invasive epithelium, to a malignant state in an invasive breast cancer free human; and/or (c) causing substantial reversion of epithelial tissue back to a normal state from an intermediate state between normal epithelium and malignant invasive epithelium.

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7   A61K31/517   A61K31/5377   A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7   A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EMBASE, CHEM ABS Data, EPO-Internal, WPI Data, PAJ, MEDLINE, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DRISCOLL D ET AL: "Effect of epidermal growth factor receptor tyrosine kinase inhibitor PD183805 on vascular endothelial growth factor secretion from several tumor models." PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH ANNUAL, vol. 40, March 1999 (1999-03), page 121 XP001014746 90th Annual Meeting of the American Association for Cancer Research;Philadelphia, Pennsylvania, USA; April 10-14, 1999, March, 1999 ISSN: 0197-016X the whole document --- -/--	1-6,8-12

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

* Special categories of cited documents :

'A' document defining the general state of the art which is not considered to be of particular relevance

'E' earlier document but published on or after the international filing date

'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

'O' document referring to an oral disclosure, use, exhibition or other means

'P' document published prior to the international filing date but later than the priority date claimed

'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

'&' document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 August 2001	27/08/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Bonzano, C

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category°	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	BASELGA J (REPRINT) ET AL: "A pharmacokinetic/pharmacodynamic trial of ZD1839 (IRESSA(TM)), a novel oral epidermal growth factor receptor tyrosine kinase (EGFR-TK) inhibitor, in patients with 5 selected tumor types (a phase I/II trial of continuous once-daily treatment)." CLINICAL CANCER RESEARCH. (NOV 1999) VOL. 5, SUPP. 'S!, PP. 29-29. PUBLISHER: AMER ASSOC CANCER RESEARCH, PO BOX 11806, BIRMINGHAM, AL 35202 ISSN: 1078-0432., XP001010516 HARPER GRACE HOSP, DETROIT, MI 48201;MD ANDERSON CANC CTR, HOUSTON, TX 77030; PETER MCCALLUM INST, MELBOURNE, VIC, AUSTRALIA; CHRISTIE HOSP, MANCHESTER, LANCS, ENGLAND; UNIV ARKANSAS, FAYETTEVILLE, AR 72701 paragraph '0028! ---	1-13
X	SMAILL J.B. ET AL: "Tyrosine kinase inhibitors. 15. 4-(Phenylamino)quinazoline and 4-(phenylamino)pyrido'd!pyrimidine acrylamides as irreversible inhibitors of the ATP binding site of the epidermal growth factor receptor." JOURNAL OF MEDICINAL CHEMISTRY, (20 MAY 1999) 42/10 (1803-1815). , XP001010518 page 1803, column 1, paragraph 1 ---	1-13
P,X	CIARDIELLO, FORTUNATO ET AL: "Antitumor effect and potentiation of cytotoxic drugs activity in human cancer cells by ZD - 1839 (Iressa), an epidermal growth factor receptor-selective tyrosine kinase inhibitor" CLIN. CANCER RES. (2000), 6(5), 2053-2063 , XP001009786 page 2054, column 1, paragraph 2 page 2059, column 1, paragraph 2 -column 2, paragraph 2 ---	1-13
X	WOODBURN J.R.: "The epidermal growth factor receptor and its inhibition in cancer therapy." PHARMACOLOGY AND THERAPEUTICS, (1999) 82/2 (241-250). , XP000965337 page 241, column 1, paragraph 1 page 246, column 1, paragraph 2 ---	1-13

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	MCKINNON C.: "Prostate cancer, biological therapy, signal transduction inhibitors and anti-angiogenesis agents." IDRUGS, (1999) 2/7 (624-628). , XP001015291 page 626, column 2, paragraph 4	1-13
X	MCKINNON C.: "Epidermal growth factor receptor targeting." IDRUGS, (1999) 2/7 (633-635). , XP001015294 page 633, column 1, paragraph 5 -column 2, paragraph 3	1-13
X	BRIDGES A.J.: "Current progress towards the development of tyrosine kinase inhibitors as anticancer agents." EMERGING DRUGS, (1998) 3/- (279-292). , XP001015295 page 282, paragraph 3 page 281, paragraph 3	1-13
A	WO 97 02266 A (CIBA GEIGY AG ;TRAXLER PETER (CH); BOLD GUIDO (CH); BRILL WOLFGANG) 23 January 1997 (1997-01-23) abstract	6,12

FURTHER INFORMATION CONTINUED FROM   PCT/ISA/ 210

Continuation of Box I.2

Present claims 1-5.8-11 relate to a compound defined by reference to a
desirable characteristic or property, namely the activity as EGFR
tyrosine kinase inhibitor.
The claims cover all compounds having this characteristic or property,
whereas the application provides support within the meaning of Article 6
PCT and/or disclosure within the meaning of Article 5 PCT for only a very
limited number of such compounds. In the present case, the claims so lack
support, and the application so lacks disclosure, that a meaningful
search over the whole of the claimed scope is impossible. Independent of
the above reasoning, the claims also lack clarity (Article 6 PCT). An
attempt is made to define the compound by reference to a result to be
achieved. Again, this lack of clarity in the present case is such as to
render a meaningful search over the whole of the claimed scope
impossible. Consequently, the search has been carried out for those parts
of the claims which appear to be clear, supported and disclosed, namely
those parts relating to the compounds mentioned in claims 6,7.

Claims searched completely: 6,7,12,13.
Claims searched incompletely:1-5,8-11.

The applicant's attention is drawn to the fact that claims, or parts of
claims, relating to inventions in respect of which no international
search report has been established need not be the subject of an
international preliminary examination (Rule 66.1(e) PCT). The applicant
is advised that the EPO policy when acting as an International
Preliminary Examining Authority is normally not to carry out a
preliminary examination on matter which has not been searched. This is
the case irrespective of whether or not the claims are amended following
receipt of the search report or during any Chapter II procedure.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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